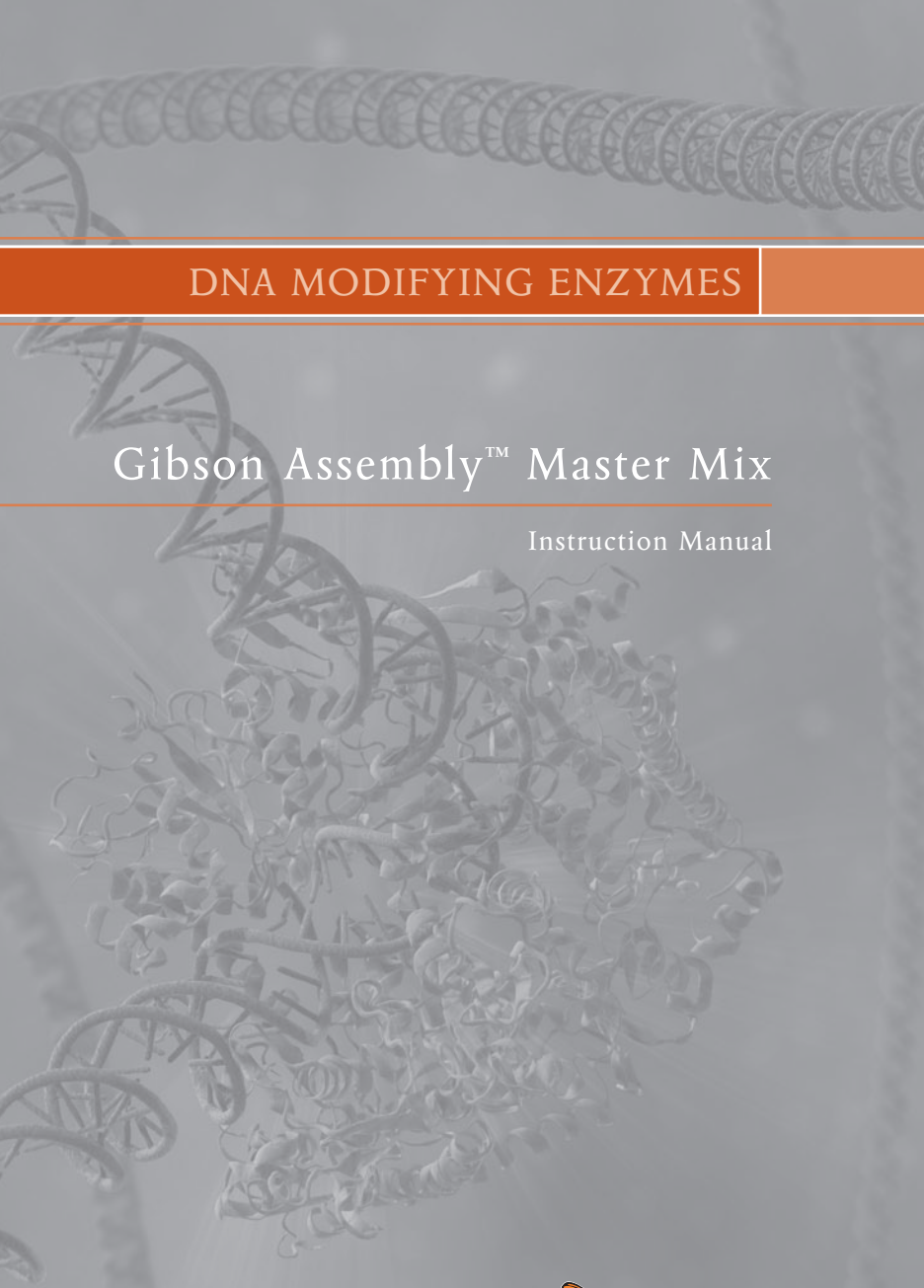
DNA MODIFYING ENZYMES

Gibson Assembly™ Master Mix

Instruction Manual



NEB #E2611S/L
10/50 reactions

NEW ENGLAND BioLabs® Inc.
enabling technologies in the life sciences

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Table of Contents:

Components:

Store at –20°C. Thaw, vortex thoroughly before use and keep on ice.

Gibson Assembly Master Mix (2X)

Positive Control

2 overlapping dsDNA fragments for control assembly.

Required Materials Not Included:

DNA Polymerases (for generating PCR products):

We recommend Phusion® High-Fidelity DNA Polymerase (NEB #M0530) or related products, such as Phusion Hot Start Flex DNA Polymerase (NEB #M0535), Phusion Hot Start Flex 2X Master Mix (NEB #M0536), Phusion High-Fidelity PCR Master Mix with HF Buffer (NEB #M0531) and Phusion High-Fidelity PCR Master Mix with GC Buffer (NEB #M0532).

LB (Luria-Bertani) plates with appropriate antibiotic.

SOC Outgrowth Medium (NEB #B9020).

Competent Cells:

We recommend NEB 5-alpha Competent *E. coli* (High Efficiency, NEB #C2987). For assembled products greater than 10 kb, NEB recommends using NEB 10-beta Competent *E. coli* (High Efficiency, NEB #C3019) or NEB 10-beta Electrocompetent *E. coli* (NEB #C3020).

Introduction:

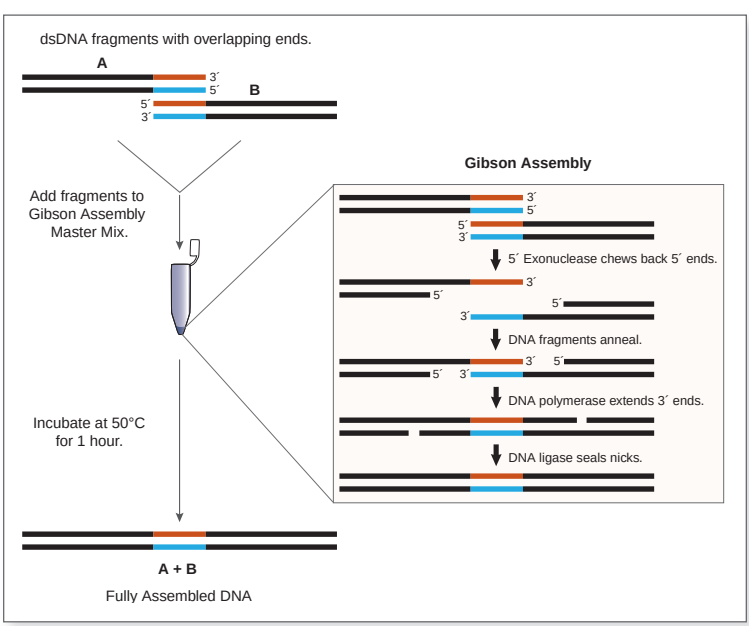
Gibson Assembly was developed by Dr. Daniel Gibson and his colleagues at the J. Craig Venter Institute and licensed to NEB by Synthetic Genomics, Inc. It allows for successful assembly of multiple DNA fragments, regardless of fragment length or end compatibility. It has been rapidly adopted by the synthetic biology community due to its ease-of-use, flexibility and suitability for large DNA constructs.

Gibson Assembly efficiently joins multiple overlapping DNA fragments in a single-tube isothermal reaction (1,2). The Gibson Assembly Master Mix includes three different enzymatic activities that perform in a single buffer:

- The exonuclease creates single-stranded 3´ overhangs that facilitate the annealing of fragments that share complementarity at one end (overlap region).
- The polymerase fills in gaps within each annealed fragment.
- The DNA ligase seals nicks in the assembled DNA.

The end result is a double-stranded fully sealed DNA molecule that can serve as template for PCR, RCA or a variety of other molecular biology applications, including direct transformation. The method has been successfully used by Gibson's group and others to assemble oligonucleotides, DNA with varied overlaps (15–80 bp) and fragments hundreds of kilobases long (1–2).

Figure 1: Overview of the Gibson Assembly Method



Specification:

10 µl of 2X Gibson Assembly Master Mix was incubated with 6 fragments (5 fragments of 400 bp and one of 2,780 bp, with 40 bp overlap, 0.05 pmol each) in a final volume of 20 µl at 50°C for 60 minutes. NEB 5-alpha Competent *E. coli* (NEB #C2987) were transformed with 2 µl of the master mix/fragment mixture using the transformation protocol on page 9. Greater than 100 white colonies were observed when 1/10 of the outgrowth was spread on an ampicillin plate with IPTG/Xgal and incubated overnight.

Overview of Gibson Assembly Master Mix Protocol:

- Design primers to amplify fragments (and/or vector) with appropriate overlaps (see pages 3–7)
- PCR amplify fragments and/or vector using a high fidelity DNA polymerase (DNA can also be prepared using a restriction digest)
- Confirm and determine concentration of fragments using agarose gel electrophoresis, a Nanodrop instrument or other method
- Add DNAs to Gibson Assembly Master Mix and incubate at 50°C for 1 hour
- Transform into *E. coli* or use directly in other applications

Design of Fragments for Assembly:

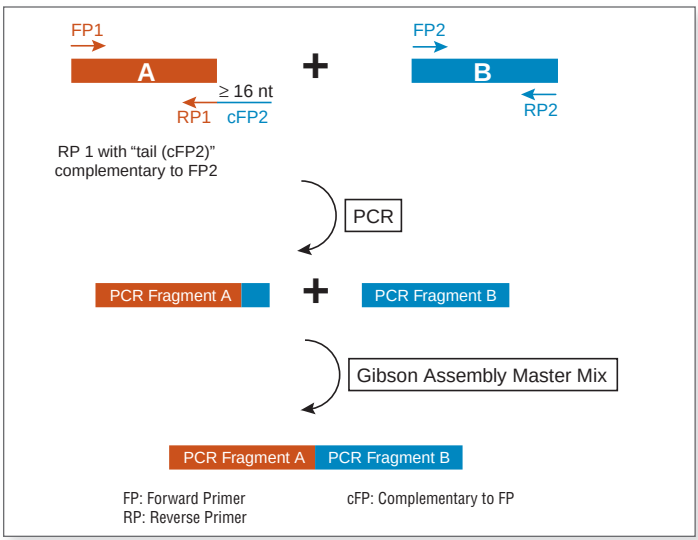
Determination of Tm for Overlap Region and PCR Primer

NEB recommends using Phusion High-Fidelity DNA Polymerase (NEB #M0530), or related products (NEB #M0535, NEB #M0536, NEB #M0531 or NEB #M0532) to amplify fragments of interest prior to assembly. While the 3´-end of the primer sequence is gene-specific, the 5´-end of the primer matches the overlap region of the two fragments to be assembled. The length of overlap region in the 5´ end of the primer is dependent on the GC content of the sequences. To achieve proper assembly of fragments, we suggest using a 16–40 nt overlap with a Tm greater than 48°C (assuming A-T pair=2°C and G-C pair=4°C). The 3´ end sequence of the primer must be specific to the gene of interest. The Tm of the 3´ gene-specific primer can be calculated using the Tm calculator found on the NEB website at http://www.neb.com/TmCalculator.

Assembly of PCR-Generated Fragments

To assemble PCR-generated fragments, the reverse primer of fragment A should be designed to include a ≥ 16 nucleotide overlapping region complementary to the 5´ end of fragment B. After PCR, the 3´ end of fragment A will overlap the 5´ end of fragment B (Figure 2).

Figure 2: Primer Design for PCR-Generated Fragment Assembly

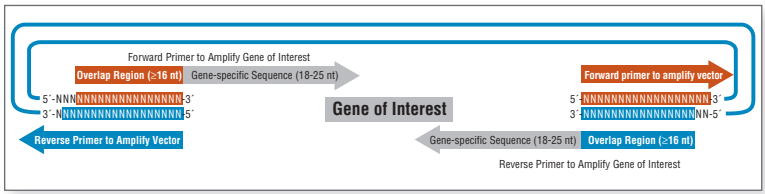


This approach can be applied to the assembly of 2 or more fragments. Additional primer design approaches that can be used to assemble fragments include adding the overlap region to the forward primer of fragment B or splitting the overlap region between the reverse primer of fragment A and the forward primer of fragment B (not shown).

Assembly of PCR-Generated Vector and Insert

Figure 3A illustrates the design of primers for a PCR-generated vector and insert to be used in assembly of a closed circular DNA molecule. Design the forward primer (orange arrow) and the reverse primer (blue arrow), specific to the vector sequence of interest. This inverse PCR strategy yields a linear vector fragment flanked by ≥ 16 nt sequences to be used in assembly with insert. Generally, 10–100 pg of a vector is recommended for inverse PCR.

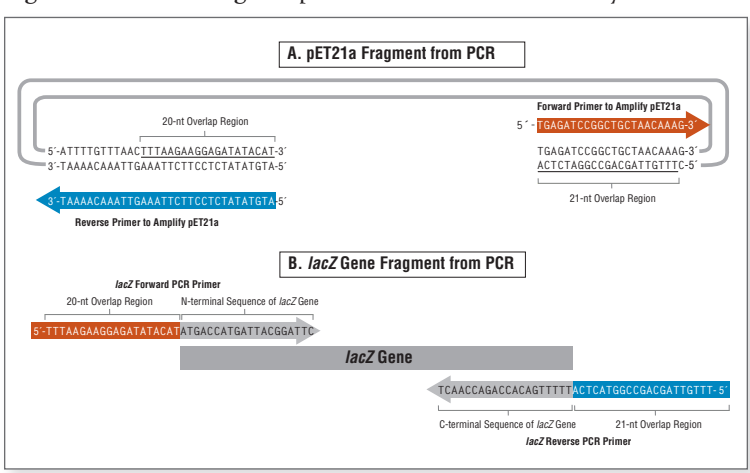
Figure 3A: PCR-Generated Vector and Insert Assembly



To amplify the insert gene of interest, design a forward primer (orange and gray) with an overlap region (≥ 16 nt, orange) at the 5´-end, which is complementary to the reverse primer (blue) of the vector. The 3´-end of the forward primer (gray) should contain gene-specific sequences to amplify the target gene. The "≥ 16 nt" overlap region (orange) of the forward primer should be complementary to the 5´-end sequence of the vector (blue). The size of the overlap region is determined by the number of nucleotides needed to reach a Tm > 48°C. If necessary, one can also add nucleotides between the overlap region and gene-specific sequence region to ensure the expressed protein is in frame. Similarly, the reverse primer (blue and gray) should also contain an overlap region (≥ 16 nt, blue) at the 5-end and gene-specific sequence at the 3´ end.

Figure 3B is an example of primer design for assembling pET21 and *LacZ* gene using this method.

Figure 3B: Primer Design for pET21a and *lacZ* Gene Assembly



Assembly of Restriction Enzyme Digested Vector and PCR-Generated Insert.

Restriction enzyme treated vectors can have 5´-overhangs, 3´-overhangs or blunt ends. The counting of a ≥ 16 nt overlap region should always start from the first nucleotide at the 3´ end, regardless of the type of overhang generated by the restriction enzyme digest (Figure 4A). The overlap region of the forward primer for the gene of interest (orange) should line up with the 3´ end of the overhang and extend back until the Tm > 48°C. This primer also includes gene-specific sequence at the 3´-end (gray). If necessary, one can also add nucleotides between the overlap region and gene-specific sequence region to ensure the expressed protein is in frame. A similar principle is applied to the design of reverse primer for the gene of interest.

Figure 4A: Assembly of Restriction Enzyme Digested Vector and PCR-derived Insert

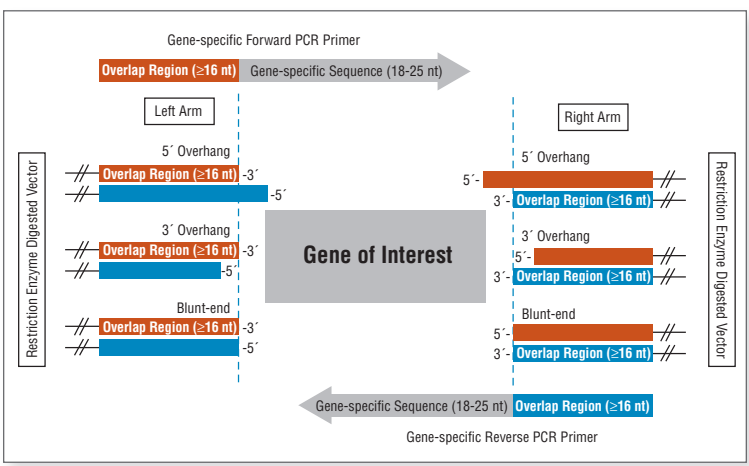
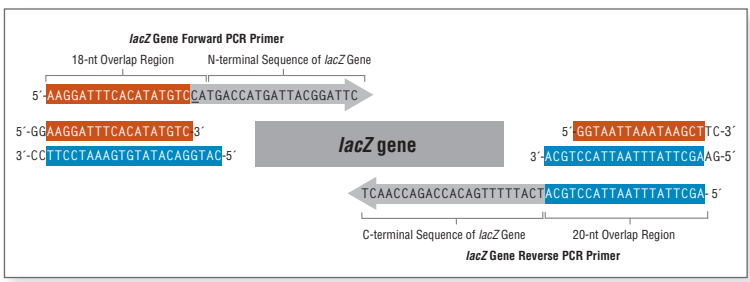


Figure 4B shows primer design for assembly of the *lacZ* gene and pMAL-c5X digested with NcoI and SbfI. In this example, the forward primer of the gene has a "C" nucleotide (underlined) inserted between the 18-nt overlap and the N-terminal sequence of the *lacZ* gene to ensure the *lacZ* protein is in frame.

Figure 4B: Primer Design for *lacZ* Gene and NcoI/SbfI-cut pMAL-c5X Assembly



Optimal Quantities:

NEB recommends a total of 0.02–0.2 pmols of DNA fragments when 2 or 3 different fragments are being assembled, and 0.2–1 pmols of DNA when 4 to 6 different fragments are used. Efficiency of assembly decreases as the number of fragments increase. To calculate the number of pmols of each fragment for optimal assembly, based on fragment length and weight, we recommend the following formula:

pmols = (weight in ng) x 1,000 / (base pairs x 650 daltons)

The mass of each fragment can be measured using the NanoDrop™ instrument, absorbance at 260 nm, or estimated from agarose gel electrophoresis followed by ethidium bromide staining.

Assembly Protocol:

1. Set up the following reaction on ice:

	Recommended Amount of Fragments Used for Assembly		
	2–3 Fragment Assembly	4–6 Fragment Assembly	Positive Control**
Total Amount of Fragments	0.02–0.2 pmols* X µl	0.2–1 pmols* X µl	10 µl
Gibson Assembly Master Mix (2X)	10 µl	10 µl	10 µl
Deionized H_2O	10-X µl	10-X µl	0
Total Volume	20 µl***	20 µl***	20 µl

* 50 ng of 5,000 bp dsDNA is about 0.015 pmols.
50 ng of 500 bp is about 0.15 pmols.
Optimized cloning efficiency is 50–100 ng of vectors with 2–3 fold of excess inserts.
Use 5 times more of inserts if size is less than 200 bps.

** Control reagents are provided for two experiments.

*** If greater numbers of fragments are assembled, additional Gibson Assembly Master Mix may be required.

2. Incubate samples in a thermocycler at 50°C for 60 minutes. Following incubation, store samples on ice or at –20°C for subsequent transformation.

3. Remove 2 µl of the assembly product and transform into competent cells of interest.

Transformation Protocol

1. Thaw competent cells on ice.
2. Transfer 50 µl of competent cells to a 1.5 ml microcentrifuge tube.
3. Add 2 µl of the chilled assembly product to competent cells. Mix gently by pipetting up and down or flicking the tube 4–5 times. Do not vortex.
4. Place the mixture on ice for 30 minutes. Do not mix.
5. Heat shock at 42°C for 30 seconds*. Do not mix.
6. Add 950 µl of room temperature SOC media* to the tube.
7. Place the tube at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
8. Warm selection plates to 37°C.
9. Spread 100 µl of the cells onto the plates.
10. Incubate overnight at 37°C.

* Please note: Follow the manufacturer's protocols for the duration and temperature of the heat shock step, as well as the optimal medium for recovery. Transformation of our positive control assembly product will yield more than 100 colonies on an Amp plate with greater than 90% colonies containing inserts.

Usage Notes:

To ensure the successful assembly and subsequent transformation of assembled DNAs, NEB recommends the following:

- *Cells*: Transformation efficiency of competent cells can vary by several logs. Perceived assembly efficiency directly correlates to the competence of the cells used for transformation.
- *Electroporation*: Electroporation can increase transformation efficiency by several logs. When using the Gibson Assembly Master Mix product for electroporation, it is necessary to dilute the reaction 3-fold and use 1 µl for transformation.
- *DNA*: Purified DNA for assembly can be dissolved in dH_2O (Milli-Q® water or equivalent is preferable), TE or other dilution buffers.
- *Insert*: When directly assembling fragments into a cloning vector, the concentration of assembly fragments should be 2–3 times higher than the concentration of vector. For assembly of 3 or more fragments, we recommend using equilmolar ratio of fragments.
- *Biology*: Some DNA structures, including inverted and tandem repeats, are selected against by *E. coli*. Some recombinant proteins are not well tolerated by *E. coli* and can result in poor transformation or small colonies.

Frequently Asked Questions (FAQs):

What should I do if my assembly reaction yields no colonies, a small number of colonies, or clones with the incorrect insert size following transformation into E. coli?

Assemble and transform the positive control provided with the Gibson Assembly Master Mix. Successful assembly of a positive control will demonstrate that the assembly mixture is functional and the transformation conditions are suitable.

Analyze the reaction on an agarose gel. An efficient assembly reaction will show assembled products of the correct size and the disappearance of fragments.

Check the primer design of the overlapping DNA fragments to ensure that there is sufficient overlap to facilitate assembly.

Consider whether the cloned insert may be toxic to *E. coli* and a low-copy vector, such as a BAC, should be used. Because the assembled product is a covalently closed molecule, it may be alternatively amplified by PCR or RCA.

What are the advantages of this method compared to traditional cloning methods?

Gibson Assembly does not rely on the presence of restriction sites within a particular sequence to be synthesized or cloned. Therefore, the user has complete control over what is assembled. Furthermore, the inclusion of unwanted additional sequence, often used to facilitate the manipulation of multiple DNA sequences, can be avoided. Lastly, a greater number of DNA fragments can be joined in a single reaction with greater efficiency than conventional methods.

How large a DNA fragment can I assemble?

Gibson Assembly has been used to assemble and clone 300 kb DNA fragments in *E. coli*, the approximate upper limit for cloning into *E. coli*. Products as large as 1 Mb have been assembled using this approach.

How many fragments of DNA can be assembled in one reaction?

The number of DNA segments that can be assembled in one reaction is dependent on the length and sequence of the fragments. Gibson Assembly has been used to efficiently assemble up to twelve 0.4 kb inserts into a vector at one time. However, we recommend the assembly of five or fewer inserts into a vector in one reaction in order to produce a clone with the correct insert. A strategy involving sequential assembly can be used if all of the fragments cannot be assembled in a single reaction.

How can I reduce the number of vector-only background colonies?

To significantly reduce the background of unwanted vector-only colonies, the vector should be a PCR product rather than a restriction fragment. If background continues to be a problem, the PCR-amplified vector can be treated with DpnI to remove the template carry-over, if applicable, extracted from an agarose gel following electrophoresis.

Can you PCR-amplify the assembled product?

Yes. The assembled DNA molecule is covalently joined and may be PCR-amplified. Additionally, if the final product is a closed circular DNA molecule, it may be used as a template in rolling-circle amplification (RCA).

What type of competent cells are suitable for transformation of DNA constructs created using Gibson Assembly?

The resulting DNA constructs are compatible with most *E. coli* competent cells. NEB recommends using NEB 5-alpha Competent *E. coli* (High Efficiency, NEB #C2987). If the assembled products are larger than 10 kb, NEB recommends using NEB 10-beta Competent *E. coli* (High Efficiency, NEB #C3019) or NEB 10-beta Electrocompetent *E. coli* (NEB #C3020).

Is this method applicable to the assembly of repetitive sequences?

Yes. However, one must ensure that each DNA fragment includes a unique overlap so that the sequences may anneal and are properly assembled. The repetitive sequence can also be internalized in the first stage of a two-stage assembly strategy. If having repetitive sequences at the ends of each fragment is unavoidable, the correct DNA assembly may be produced, albeit at lower efficiency than other, unintended assemblies.

What are the shortest overlaps that can be used with this assembly method?

Productive assembly has been shown for DNA fragments with as little as 12 bp overlap. However, it depends on the GC content of the overlap. We recommend using 16 bp overlaps or more for dsDNA assembly with a T_m > 48°C (AT pair = 2°C and GC pair = 4°C).

What are the longest overlaps that can be used with this method?

The quantity of exonuclease in the Gibson Assembly Master Mix has been optimized for the assembly of DNA molecules with ≤ 100-bp overlaps.

Is it necessary to gel-extract restriction fragments or PCR products?

Gel-extraction of restriction fragments is generally not necessary. A column cleanup kit or a standard phenol-chloroform extraction, followed by ethanol precipitation is sufficient. We have also used unpurified PCR products directly in assembly reactions as long as the PCR product is > 90% pure.

Can ≤ 200 bp dsDNA fragments be assembled by this method?

Yes. For optimal results, use these fragments in ≥ 5-fold excess.

Can ssDNA oligonucleotides be combined and assembled with dsDNA fragments?

Yes. However, the optimal concentration of each oligonucleotide should be determined. As a starting point, we recommend using 45 nM of each oligonucleotide that is less than or equal to twelve 60-base oligonucleotides containing 30-base overlaps.

Can longer or shorter incubation times be used?

Yes. The Gibson Assembly Kit has been optimized for assembly in 1 hour at 50°C. However, in some cases, 15 minutes may be sufficient. Reaction times less than 15 minutes are generally not recommended. Extended incubation times (e.g., 2–16 hours) have been shown to improve assembly efficiencies in some cases.

Will the reaction work at other temperatures?

The reaction has been optimized at 50°C, but it has been shown to work between 40°C and 50°C.

I would like to produce overlapping dsDNA fragments by PCR. Do I need to use PCR primers that have been purified by PAGE or HPLC?

No. Standard, desalted primers may be used.

I would like to assemble ssDNA oligonucleotides into dsDNA fragments. Do I need to use oligonucleotides that have been purified by PAGE or HPLC?

No. Standard, desalted primers may be used.

References:

1. Gibson, D.G. et.al. (2009) *Nature Methods,* 343–345.
2. Gibson, D.G. et al. (2010) *Nature Methods*, 901–903.

For additional references and information on Gibson Assembly Master Mix, visit www.syntheticgenomics.com. and www.jcvi.org.

Ordering Information

PRODUCT	NEB #	SIZE
Gibson Assembly Master Mix	E2611S/L	10/50 reactions
COMPANION PRODUCTS		
Phusion High-Fidelity DNA Polymerase	M0530S/L	100/500 units
Phusion Hot Start Flex DNA Polymerase	M0535S/L	100/500 units
Phusion Hot Start Flex 2X Master Mix	M0536S/L	100/500 rxns
Phusion High-Fidelity PCR Master Mix with HF Buffer	M0531S/L	100/500 rxns
Phusion High-Fidelity PCR Master Mix with GC Buffer	M0532S/L	100/500 rxns
NEB 5-alpha Competent *E. coli* (High Efficiency)	C2987I/H	6 x 0.2 ml/ 20 x 0.05 ml
NEB 10-beta Competent *E. coli* (High Efficiency)	C3019I/H	6 x 0.2 ml/ 20 x 0.05 ml
NEB 10-beta Electrocompetent *E. coli*	C3020K	6 x 0.1 ml
SOC Outgrowth Medium	B9020S	4 x 25 ml

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